Laser Photolysis Behavior of Ferrous Horseradish Peroxidase with Carbon Monoxide and Cyanide: Effects of Mutations in the Distal Heme Pocket†

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ABSTRACT: Native horseradish peroxidase and several forms with mutations in the distal heme pocket (His42Leu, His42Arg, and Arg38Leu) have been expressed in *Escherichia coli.* These enzymes have been purified and analyzed in terms of the room temperature recombination rate of carbon monoxide and cyanide after photolysis of the reduced forms. The recombinant wild-type ferrous form exhibited monophasic recombination of carbon monoxide with an observed bimolecular rate constant at pH 8.5 of 4.4×10^3 M^{-1} s^{-1} which is essentially the same as the natural glycosylated form. This recombination rate constant increases in the mutants in the order WT < H42R < H42L ≪ R38L. The value for R38L (5×10^6 M^{-1} s^{-1}) is increased by 3 orders of magnitude relative to the wild-type and is similar to that for human hemoglobin [Mims et al. (1983) *J. Biol. Chem. 258,* 14219−14232]. Cyanide recombination with the wild-type ferrous form at room temperature is biphasic at pH 6.5 but becomes more monophasic at pH 8.5, again similar to the behavior of the natural glycosylated form, although the Fe^{2+}−cyano form of the recombinant enzyme appears to be more unstable at high pH. None of the mutant forms were able to bind cyanide in the ferrous state to any significant extent (K_{diss} > 250 mM) when cyanide was added at a concentration (10−20 mM) sufficient to almost saturate the wild-type form (K_{diss} ≅ 1 mM at pH 7). This behavior contrasts with that of the oxidized forms of the mutants where increases in cyanide dissociation constants are smaller (<25 times). The results are rationalized in terms of the polarity of the route of access and binding site of carbon monoxide and cyanide, and the need for a charge-compensating protonation site for the stable binding of the cyanide anion, but not for the binding of the neutral carbon monoxide.

Horseradish peroxidase (HRP)[1] and its reactions with classical heme ligands are relatively well documented. Two such ligands are carbon monoxide and cyanide. The photolysis and subsequent recombination of carbon monoxide with ferrous HRP have been reported in detail (Coletta et al., 1986; Berinstain et al., 1990). Recombination behavior is only weakly dependent upon pH in the pH range 3−11, with a room temperature recombination rate constant of (3−4) × 10^3 M^{-1} s^{-1} at pH 7.0. Cyanide binding to oxidized HRP is also well understood, and results in the formation of a typical low-spin ferric heme compound (Blumberg et al., 1968; Dunford & Nadezhdin, 1982). The reaction of cyanide with reduced HRP has been less well studied. Although the ferroheme−cyanide compound has been known since the studies of Keilin and Hartree (Keilin & Hartree, 1955; Blumberg et al., 1968), we know of only one paper in which this reaction has been studied in some detail (Phelps et al., 1971). A dissociation constant of around 1 mM at pH 7 was found, and its lack of pH-dependency around pH 7 suggests that the net species bound was HCN. Like the carbon monoxide compound, the ferroheme−cyanide compound is also photolabile (Hill & Marmor, 1991), but this property does not appear to have been exploited. Photolysis studies of the analogous cyanide compounds of cytochrome oxidase (Hill & Marmor, 1991) and bacterial cytochrome *bo* have been reported (Mitchell et al., 1995).

We have recently noted a rule of local electroneutrality of ligand and redox state changes for the binuclear center of protonmotive oxidases, in which binding of any charged species is compensated by protonation so that local electroneutrality is maintained (Mitchell & Rich, 1994). This principle has allowed the definition of central features of proton/electron coupling in this class of enzymes (Rich, 1995). It was of interest to know whether this might be a consideration in other high-spin protoheme systems. The availability of site-directed mutants of HRP offers a means of extending such studies to an enzyme with a relatively well-defined structure.

Although a high-resolution crystal structure of HRP is not yet available (Henriksen et al., 1995), the structure around its heme group can be predicted with some confidence because of the homology with other peroxidases (Welinder, 1992), such as *Cc*P whose structure is available at atomic resolution (Finzel et al., 1984; Edwards et al., 1984). Model building on this basis has suggested that the core of HRP and *Cc*P and their active sites are conserved (Smith et al., 1995; Loew et al., 1995). In the absence of direct crystal-

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[1] Abbreviations: HRPC, horseradish peroxidase isoenzyme C; HRPC*, recombinant horseradish peroxidase isoenzyme C; R38L, Arg38Leu HRPC* mutant; H42L, His42Leu HRPC* mutant; H42R, His42Arg HRPC* mutant; *Cc*P, cytochrome *c* peroxidase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CO, carbon monoxide; K_{diss}, dissociation constant.

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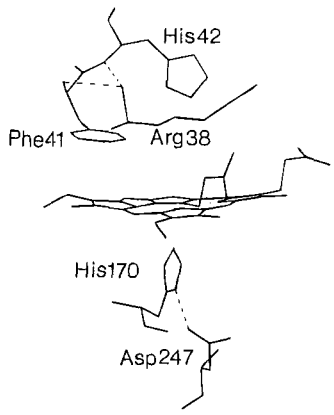

FIGURE 1: Key residues in the ligand binding site of HRPC. The catalytic residues of HRP discussed in this work are shown as they appear in the CcP (W51F) crystal structure. Residues are numbered according to the HRP sequence.

lographic information for HRPC, ^{1}H NMR studies (La Mar et al., 1992) indicate that several key catalytic residues have a similar disposition relative to the heme to that found in CcP (Figure 1). In myoglobins and hemoglobins, a distal histidine residue at position 64 is thought to play a pivotal role in determining the binding of diatomic ligands to the heme iron. Comparison of the binding constants and the structures of CO bound to model compounds and native proteins has led to the hypothesis that the binding of CO is modulated by interactions with the distal histidine (Balasubramanian et al., 1993a; Ray et al., 1994). However, examination of mutations of other amino acids on the distal side of the heme pocket indicates that other nearby residues can affect the accessibility and orientation of bound ligands (Balasubramanian et al., 1993a,b, 1994; Cameron et al., 1993; Gibson et al., 1992; Li et al., 1994; Smerdon et al., 1995; Quillin et al., 1995). Peroxidases also possess an equivalent distal histidine residue, but a catalytic arginine takes the place of the distal phenylalanine residue that is found in myoglobins and hemoglobins (Poulos & Finzel, 1984). Ligand binding properties of a variety of mutations of residues in the distal heme pocket of CcP have been reported (Miller et al., 1990a,b, 1992, 1994; Smulevich et al., 1991; Erman et al., 1993; Vitello et al., 1993). The recent development of site-specific mutagenesis in HRP has made it possible to engineer analogous changes in the distal heme pocket to investigate how these residues modulate heme reactivity. This study describes the role of the distal His and Arg in the reaction of HRP with carbon monoxide and cyanide. Our data are compared with the reported effects of comparable mutations that have been made in CcP and in myoglobin, providing further insight at the atomic level of the different behavior of two heme proteins groups, peroxidases and globins. This offers the possibility of engineering new proteins from peroxidases with modified O_2 binding properties [cf. Miller et al. (1994)].

MATERIALS AND METHODS

Construction of Mutant HRPC Genes.* A polymerase chain reaction (PCR)-based technique with the HRPC synthetic gene (Smith et al., 1990) as template was used to generate insert DNA bearing the required mutations. For the construction of the three mutants used in this study, R38L, H42L, and H42R, the polymerase chain reaction was performed as previously described for the Phe41Val mutation (Smith et al., 1992), except that oligonucleotide V1 was replaced by N1, N3A, or N3B, respectively. N1 was identical to the wild-type synthetic gene sequence between nucleotide positions 111 and 147, except that it contained the Leu codon TTA at position 126 (5'GCTGCTTCAATAT-TATTACT-GCACTTCCATGAC3'). N3A and N3B contained the Leu codon CTC and the Arg codon CGC at position 138, respectively. Amplified DNA fragments (404 bp) bearing the mutation were cloned in-frame into the wild-type gene at the unique *Ssp*I and *Xho*I sites in the HRP expression vector pAS5 (Smith et al., 1992). Double-stranded DNA sequence analysis by the dideoxy chain termination method, using sequenase version 2 (Saunders & Burke, 1990) and oligonucleotides S1 and N1, N3A, or N3B (Smith et al., 1992) as sequencing primers, confirmed the expected sequence change.

Preparation of Peroxidase. HRP (EC 1.11.1.7) isoenzyme C was purchased from Sigma Chemical Co. (type VI) and used without further purification. Growth and induction of *E. coli* strains producing recombinant peroxidase variants were as previously described (Smith et al., 1992). Folding and activation of HRPC* recovered from *E. coli* inclusion bodies were achieved essentially by the method of Smith et al. (1990) with the modifications subsequently described in Smith et al. (1992). Purified enzyme was desalted into 10 mM Na-MOPS (pH 7.0) and stored in liquid nitrogen until used. The concentration of the enzyme was determined using the Soret extinction coefficient determined by the pyridine hemochromogen method (Furhop & Smith, 1975). These were 102 mM^{-1} cm^{-1} for HRPC and HRPC*, 86 mM^{-1} cm^{-1} for R38L, 96.5 mM^{-1} cm^{-1} for H42L, and 88 mM^{-1} cm^{-1} for H42R. The Rz (Reinheitszahl) values of the HRP preparations used in this work were 3.2 for HRPC, HRPC*, R38L, and H42R and 3.4 for H42L.

Photolysis of Fully Reduced, Cyanide-Ligated Enzyme. Fully reduced HRP was formed by incubation of enzyme with solid sodium dithionite for several minutes in a buffer of 0.2 M potassium phosphate at pH 6.5 or 0.2 M Tris-HCl at pH 8.5, until no further development of the reduced Soret band could be detected. Cyanide was added from a stock 2 M solution which had been neutralized before use. The cuvette was sealed to avoid loss of HCN. Room temperature photolysis was achieved with a flash from a frequency-doubled Nd-YAG laser (Spectron Ltd., Rugby, U.K.). This produced 10 ns pulses of light at 532 nm with energy in excess of 100 mJ/pulse. The photomultiplier was protected with appropriate filters to avoid flash artifacts. Transients were recorded separately at several wavelengths and signal-averaged if necessary. Kinetic spectra at various times after the flash were reconstructed from these transients as described previously (Brown et al., 1994).

Photolysis of Fully Reduced, Carbon Monoxide-Ligated Enzyme. The sample was treated as for cyanide above, but the cyanide addition was replaced by gassing of the samples for several minutes with pure carbon monoxide. Data were collected as for cyanide photolysis.

RESULTS

Photolysis and Recombination of Carbon Monoxide. The photolability of the carbon monoxide compounds of ferrous hemes is well-known (Huang et al., 1991), and the reaction

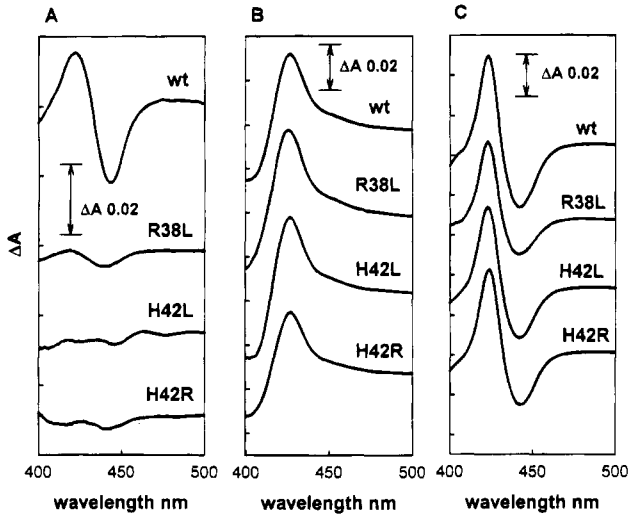

FIGURE 2: Comparison of ligand-induced binding spectra of wild-type and mutant forms of HRPC*. Enzymes were dissolved to a concentration of 0.8 μM in 0.5 mL of a buffer of 0.2 M potassium phosphate and 2 mM EDTA at pH 6.5 (panels A and B) or 0.2 M Tris-HCl and 2 mM EDTA at pH 8.5 (panel C). Samples in panels A and C were reduced with solid sodium dithionite, and a base line was recorded. The difference spectra shown were recorded after addition of 20 mM KCN (panel A), 1 mM KCN (panel B), or saturating carbon monoxide (panel C). Hence, difference spectra are (panel A) (reduced *plus* 20 mM cyanide) *minus* (reduced), (panel B) (oxidized *plus* 1 mM cyanide) *minus* (oxidized), and (panel C) (reduced *plus* carbon monoxide) *minus* (reduced).

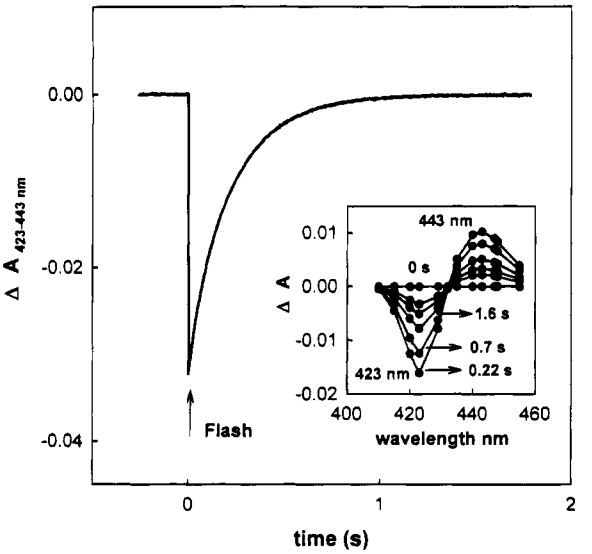

FIGURE 3: CO recombination after laser flash photolysis and photolysis spectra of the wild-type HRPC*. The enzyme was dissolved to a concentration of 0.8 μM in 0.5 mL of a medium containing 0.2 M Tris-HCl and 2 mM EDTA at pH 8.5. The solution was reduced with solid sodium dithionite, saturated with carbon monoxide by bubbling, and sealed against gas exchange. Laser photolysis and recombination were monitored separately at 423 and 443 nm without signal-averaging, and the difference 423 − 443 nm is plotted. The insert shows the photolysis spectra at different times after the flash, and the spectra were computed from transient data taken at many different wavelengths.

in ferro-HRP has been described in detail (Coletta et al., 1986). All mutant forms were able to bind carbon monoxide in their reduced forms (Figure 2C). In order to compare their kinetic behavior, we restricted our measurements to photolysis and recombination at pH 8.5. The behavior of the recombinant enzyme is shown in Figure 3. After photolysis, the carbon monoxide recombined monophasically with an observed rate constant of around 4.4 s^{-1} at 1 atm of carbon monoxide. The photolysis spectra taken at various times after the flash (inset to Figure 3) were an exact inverse of the carbon monoxide binding spectrum, with a peak and trough at 443 and 423 nm, respectively, and showed only a single optical component. This behavior is essentially identical to that of the natural glycosylated form of the enzyme (data not shown) and corresponds well with previous reports (Coletta et al., 1986). The mutant forms all had faster recombination rates (Table 1 and Figure 4). The smallest effect was observed for H42R, which recombined monophasically with a k_{obs} of 8.3 s^{-1}. The other two mutants showed biphasic recombination kinetics. Both phases of recombination with H42L were faster than H42R, and recombination rates with R38L were very fast (k_{obs} of 5000 and 200 s^{-1}).

Cyanide Binding to Ferrous HRP. Cyanide bound to the reduced form of ferrous HRP with a K_{diss} value of approximately 1 mM (Phelps et al., 1971) and gave a characteristic binding spectrum. However, although all mutant enzymes were readily reduced by sodium dithionite, and could bind carbon monoxide normally (Figure 2C), addition of 20 mM cyanide to their reduced forms at pH 6.5 failed to induce the spectral change induced in the wild-type (Figure 2A), indicating that they no longer bound cyanide significantly at this concentration. In all cases, the K_{diss} was estimated at ≥200 mM. Even the small changes observed, for example on addition of cyanide to ferrous R38L

Table 1: Kinetic Parameters of CO and CN Recombination after Flash Photolysis[a]

enzyme	fast phase k_{obs} (s^{-1})	slow phase k_{obs} (s^{-1})	fraction as fast phase	isosbestic point (nm)[b]
(A) CO Recombination Kinetics (1 mM CO; pH 8.5; 423−443 nm)				
HRPC	4		1.0	432
HRPC*	4.4		1.0	432
R38L	5000	200	0.55	430
H42L	60	13	0.7	431
H42R	8.3		1.0	433
(B) Cyanide Recombination Kinetics (10 mM Cyanide; pH 8.5; 432−448 nm)				
HRPC	1.4	0.23	0.57	
HRPC*	0.68	0.24	0.55	
(C) Cyanide Recombination Kinetics (5 mM Cyanide; pH 6.5; 432−448 nm)				
HRPC	3	0.1	0.55	
HRPC*	2.4	0.12	0.4	

[a] The samples were prepared as described in Figures 3, 4, and 5. Laser photolysis and ligand recombination were monitored as in Figures 3, 4, and 5. [b] Isosbestic points were determined from the photolysis spectra at different times after the flash.

(see Figure 2A), are likely to arise from oxidation and binding of cyanide to the ferric state, a change which induces an almost identical spectral shift in this system (data not shown). The decreased ability to form the ferrous cyanide compound was investigated further by examination of the photolysis behavior, since the ferrous cyanide compound, but not the ferric cyanide compound, is photolabile.

Photolysis and Recombination of Cyanide with Ferrous HRP. The photolysis and recombination of ferrous HRPC* with cyanide have not been described in any detail to our knowledge. The behavior of the recombinant wild-type enzyme at pH 6.5 and 8.5 is therefore shown in Figure 5. The photolysis yield was approximately 30% at pH 8.5

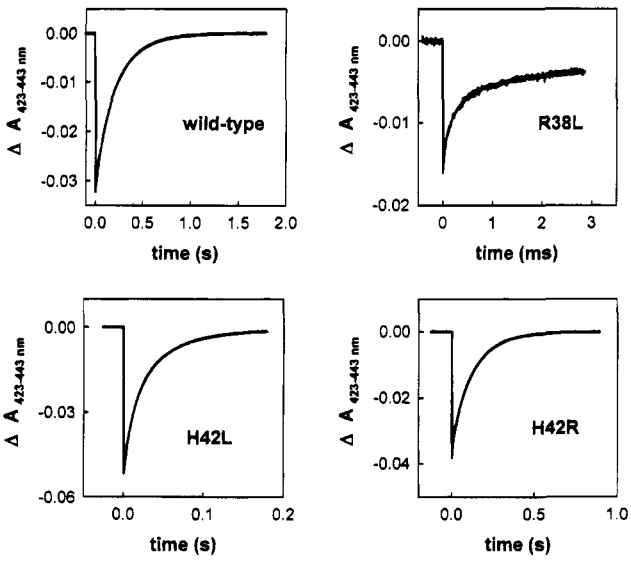

FIGURE 4: CO recombination after laser flash photolysis of the wild-type and mutant forms of HRPC*. Samples were prepared as described in Figure 3. In order to increase signal/noise for the R38L mutant, 10 transients were taken and signal-averaged at each wavelength. For other forms, no averaging was performed. The trace for R38L shows only the fast phase of recombination (5000 s^{-1}) on this time scale, but the remainder relaxes back to the base line on longer time scales with a 200 s^{-1} rate constant.

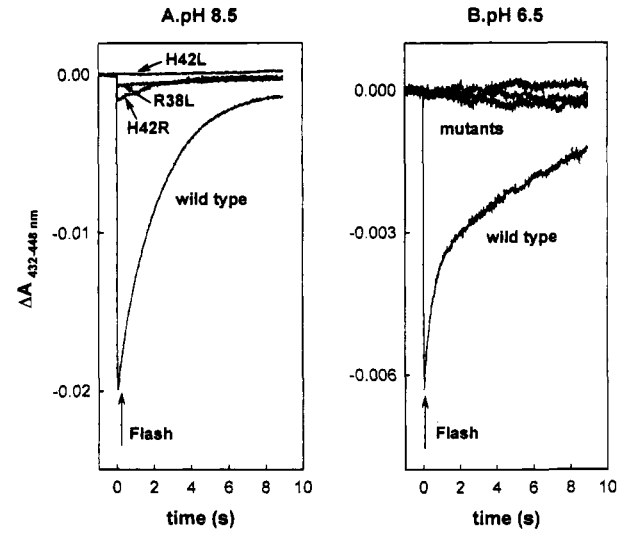

FIGURE 5: Cyanide recombination after laser flash photolysis of wild-type and mutant forms of HRPC*. The enzymes were dissolved to a concentration of 0.8 μM in 0.5 mL of a buffer containing 0.2 M Tris-HCl and 2 mM EDTA at pH 8.5 (panel A) or 0.2 M potassium phosphate and 2 mM EDTA at pH 6.5 (panel B). They were reduced with solid sodium dithionite, and either 10 mM KCN (pH 8.5, panel A), 5 mM KCN (wild-type at pH 6.5, panel A), or 20 mM KCN (mutants at pH 6.5, panel B) was then added. Photolysis and recombination of cyanide were monitored at 432–448 nm, and the data were obtained by collection of a single transient separately at each wavelength.

and 8% at pH 6.5, and recombination was biphasic. This biphasicity was most acute at the lower pH, with more than a factor of 10 difference between the fast and slow phases (Table 1). At least at pH 6.5 the two phases are optically distinct, and only the slower phase corresponds to the inverse of the static spectrum of binding of cyanide to the reduced enzyme. The detailed interpretation of these complex kinetics and spectra will be described elsewhere.

Table 2: Dissociation Constants of the Ferric-Cyanide Compounds of HRP Mutants[a]

enzyme	K_{diss} (μM)
HRPC*	3
R38L	65
H42L	30
H42R	10

[a] Enzymes were dissolved in 0.5 mL of 0.2 M potassium phosphate and 2 mM EDTA at pH 6.5 to a final concentration of 0.8 μM. Successive additions of neutralized potassium cyanide were made, and the optical changes due to formation of the cyanide compound were monitored at 426–400 nm. The K_{diss} was estimated as the concentration of cyanide necessary to obtain the half-maximal signal.

The behavior of the natural glycosylated enzyme was similar (data not shown, rate constants listed in Table 1). We did however notice that the recombinant protein was rather unstable at pH 8.5 when in its reduced state in the presence of cyanide, and appeared to be made worse by the EDTA in the buffer. This instability was not apparent in the natural glycosylated form, and its origin is presently unknown. As predicted from the lack of binding spectra, none of the three mutant enzymes exhibited any cyanide-induced photolysis signals when incubated with at least 10 mM cyanide at pH 8.5 or 20 mM cyanide at pH 6.5. The very small transients seen in some instances are present even before cyanide addition. They relax back to the origin on longer time scales and so are reversible, but we did not investigate further their possible chemistry.

Cyanide Binding to Ferric HRP. The lack of cyanide binding to the reduced forms of the mutant enzymes is in contrast to their retention of the ability to bind cyanide in their oxidized forms. In all mutants, cyanide induced an optical change characteristic of the ferric heme–cyanide adduct (Figure 2B). Titration of the dissociation constant of cyanide by monitoring the extent of formation of the optical signal at different cyanide concentrations showed that, although binding was weakened in oxidized mutant forms, the dissociation constants had increased much less dramatically than was the case for their reduced forms (Table 2).

DISCUSSION

A variety of effects of mutations on some analogous ligand binding reactions of myoglobin (Balasubramanian et al., 1993a,b, 1994; Cameron et al., 1993; Gibson et al., 1992; Li et al., 1994; Smerdon et al., 1995; Quillin et al., 1995) and *Cc*P (Miller et al., 1990a,b, 1992, 1994; Smulevich et al., 1991; Erman et al., 1993; Vitello et al., 1993) have been reported. In these papers, the importance of ligand access routes, steric factors, electrostatics, bound water, and hydrogen bonding has been stressed as possible influences that can affect association and dissociation rate constants and affinities. Also important is a consideration of any protonation changes associated with ligand binding. Relevant data on protonation changes associated with ligand binding have been reported, for example, for HRP (Dunford & Stillman, 1976; Yamada & Yamazaki, 1974), *Cc*P (DeLauder et al., 1994; Dunford & Alberty, 1967; Ellis & Dunford, 1968), catalase and myoglobin (Millar et al., 1981), and the iron–copper terminal oxidases (Mitchell & Rich, 1994).

Carbon monoxide binding to ferrous HRP is likely to occur with no net protonation change, which simplifies possible interpretations of mutation effects. Replacement of the distal

His42 by Leu or Arg produced a 15- and 2-fold increase in the recombination rate, respectively, whereas the replacement of the distal Arg38 by leucine caused a far more dramatic increase (Table 1). These effects of mutations on the CO association rate constant can be compared with other systems in which the reaction and the effects of mutations have been studied. For example, the reaction has been studied in mutant forms of myoglobin, including mutants in which the distal His64 was variously replaced (Li et al., 1994; Smerdon et al., 1995). Roughly similar factors of increase in the recombination rates were found for similar types of replacements [for example, an increase of ×52 for His64Leu and ×2 for His64Gln (Li et al., 1994)]. These results suggest that, as in myoglobins and hemoglobins, the distal histidine impedes the binding of CO to HRP. However, it should be noted that the recombination rate constant of wild-type HRP is strikingly low [$(3-4) \times 10^3$ M^{-1} s^{-1}] when compared to that of wild-type myoglobin [e.g., sperm whale myoglobin has $k = 5 \times 10^5$ M^{-1} s^{-1} (Mims et al., 1983)] and hemoglobin [R-state human hemoglobin has $k > 10^6$ M^{-1} s^{-1} (Mims et al., 1983; Babcock & Varotsis, 1993)]. In these globins, there is no equivalent of the distal Arg38 of HRP, the position being occupied instead by Phe43. Hence, the observed increase of 3 orders of magnitude in the rate of CO recombination for the Arg38 → Leu mutant of HRP, converting its behavior to resemble the globins, suggests that the low rate of reaction of HRP with CO is primarily the result of a more hindered access route for this ligand to the heme iron, perhaps being exacerbated by a more polar nature of the channel [cf. discussion in Mims et al. (1983)].

A comparison with the results from analogous studies of mutant forms of *Cc*P is also of interest. The structures of the distal heme pocket of HRP and *Cc*P are likely to resemble each other more closely than they resemble the globins (Smith et al., 1995; Loew et al., 1995), particularly in that both have the distal arginine next to the distal histidine. However, *Cc*P displays a number of properties that are quite different from HRP, making a detailed comparison of mutation effects rather difficult. Of particular relevance to our studies is the presence of acidic and basic forms of *Cc*P with a pK of interconversion around 8 (Iizuka et al., 1985; Miller et al., 1990a), and with the two forms displaying radically different rate constants of CO recombination. Only the acid form displays a CO recombination rate constant [$(1-2) \times 10^3$ M^{-1} s^{-1}] which is similar to that of HRP. The effects of mutation of the distal Arg38Leu of *Cc*P on CO recombination behavior have been studied, and, in contrast to our observations with HRP, it was concluded that mutation of this residue had little effect on CO recombination rate constants of either the acidic or the basic form of *Cc*P (Miller et al., 1990a). However, it was noted in this report that full formation of the slowly-recombining acid form of the Arg38Leu mutant could not be achieved, implying a heterogeneity of some kind and making it impossible for us to take this comparison further.

Neither cyanide binding to ferrous HRP nor its photolysis behavior has been widely studied. The cyanide compound of ferrous *Cc*P has been long known, and is similarly photolabile (Meunier and Rich, unpublished data), but it also has received little attention. In addition, studies of the cyanide compounds of ferrous hemoglobin and ferrous myoglobin are limited since they have very high dissociation constants such that they can only be studied in the presence of very high cyanide concentrations (Keilin & Hartree, 1955) or as transient species after reduction of the ferric−cyanide forms (Brunori et al., 1992). The photolysis and recombination behavior of cyanide with ferrous HRP is pH-dependent and biphasic (Figure 5). The detailed analysis and interpretation of this complex behavior, which does not arise from enzyme heterogeneity, will be reported elsewhere. However, the most important finding with all three mutants is that none are able to bind cyanide in their reduced forms. Even the small photolysis changes observed in the mutants at pH 8.5 are unlikely to arise from a cyanide compound since they are present even in the absence of added cyanide and all K_{diss} are estimated to be ≥200 mM (cf. 1 mM in the wild-type form). In contrast, all mutants can still form ferric−cyanide compounds with K_{diss} values within a factor of 25 of the wild-type value (Table 2).

The binding of cyanide to oxidized and reduced forms of HRP and *Cc*P is known to involve a protonation; i.e., HCN, rather than CN^-, is bound overall (Dunford & Stillman, 1976; Phelps et al., 1971; Erman, 1974). However, since the cyanide binds to the heme iron *via* its carbon atom, the proton must be displaced from the carbon of the cyanide, onto a protonation site close enough to compensate the resultant negatively charged cyanide anion. The crystal structure studies of the cyanide and fluoride complexes of oxidized *Cc*P (Edwards & Poulos, 1990) show that this protonation site is provided by the distal histidine. However, the pH-dependency of the midpoint potential of HRP (Harbury, 1957), at least below pH 7.4, shows that there is also a redox-linked protonation site with a pK on the ferrous form at this pH, and this is most likely to be provided by the same distal histidine (Kitagawa & Teraoka, 1982) in the wild-type enzyme. This is also likely to be the case in *Cc*P, where it has been shown that the low pK of the distal histidine in the ferric enzyme rises on anion binding to (Smulevich et al., 1991) (or, presumably, electronation of) the heme group and so would provide the anion- or redox-linked protonation site. Hence, the conversion of oxidized HRP into the reduced, cyanide-ligated state can require two protonation sites, one for the electron and one for the cyanide anion. The proximal histidine is always hydrogen-bonded to the side chain of Asp247 (Figure 1) and so cannot provide a second protonation site. Instead, it seems most likely that the arginine and associated water molecules in the distal site could provide the alternative protonation site, perhaps formally existing as an H_3O^+ in this polar network. The important role of the arginine in hydrogen-bonding stabilization of the fluoride and cyanide ferric compounds has been clearly shown in the crystal structures of these ligand complexes of *Cc*P (Edwards & Poulos, 1990). The dramatic decrease in cyanide affinity of the ferrous forms of the mutants may therefore arise primarily from a lack of appropriate sites for cyanide-linked protonation. The effect of these mutations on cyanide binding to the oxidized forms is less dramatic, presumably since a redox-linked decrease in the number of available protonation sites has not occurred.

The overall changes in the binding of cyanide to the oxidized and reduced forms can therefore be rationalized in terms of the limited availability of charge-compensating protonation sites, together with changes in the polarity of the binding pocket and channel. Since CO binds to ferrous heme without the need for charge-compensation, only the latter factor influences its binding rate (and, presumably,

binding constant). Mutagenesis of two distal residues, His42 and Arg38, in HRP differentially modulates the binding of CO and HCN to the heme. The reassociation rates of CO approach those previously reported for sperm whale myoglobin and human hemoglobin (Mims et al., 1983; Babcock & Varotsis, 1993), especially in the R38L mutant. Binding of cyanide to the ferrous forms of all the mutants becomes very weak, as in the globins. In addition, it has recently been shown that the R38L mutation in HRP decreases the apparent bimolecular rate constant for the reaction of the ferric enzyme with H_2O_2 by 3 orders of magnitude (Rodriguez-Lopez et al., unpublished data). Thus, a single substitution of a positive-charged residue (Arg38) by an apolar one (Leu) in HRP causes a reactivity pattern that is much more similar to that observed for hemoglobins and myoglobins, demonstrating that the differences between peroxidases and globins can in part be explained in terms of the different polarity of their respective distal heme pockets, although secondary effects on the degree of electroneutrality of the heme iron, modulated by protein environment, cannot be ruled out as a causal factor in modulating the rates of ligand binding.

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